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In Vivo Formation and Persistence of Modified Nucleosides Resulting From Alkylating Agents

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Alkylating agents are ubiquitous in the human environment and are continuously synthesized *in vivo*. Although many classes exist, interest has been focused on the *N*-nitroso compounds, since many are mutagens for bacteria, phage, and cells, and carcinogens for mammals. In contrast to aromatic amines and polyaromatic hydrocarbons which can react at carbons, simple alkylating agents react with nitrogens and oxygens: 13 sites are possible, including the internucleotide phosphodiester. However, only the *N*-nitroso compounds react extensively with oxygens. *In vivo*, most possible derivatives have been found after administration of methyl and ethyl nitroso compounds. The ethylating agents are more reactive toward oxygens than are the methylating agents and are more carcinogenic in terms of total alkylation. This is true regardless of whether or not the compounds require metabolic activation. It has been hypothesized that the level and persistence of specific derivatives in a "target" cell correlates with oncogenesis. However, no single derivative can be solely responsible for this complex process, since correlations cannot be made for even a single carcinogen acting on various species or cell types. Some derivatives are chemically unstable, and the glycosyl bond is broken (3- and 7-alkylpurines), leaving apurinic sites which may be mutagenic. These, as well as most adducts, are recognized by different enzymatic activities which remove/repair at various rates and efficiencies depending on the number of alkyl derivatives, as well as enzyme content in the cell and recognition of the enzyme. Evaluation of human exposure requires early and sensitive methods to detect the initial damage and the extent of repair of each of the many promutagenic adducts.

Introduction

Although one of the earliest presumed carcinogens, chimney soot, contains polyaromatic hydrocarbons, the study of reaction of aromatic carcinogens with cellular compounds was long handicapped by lack of knowledge of metabolites, making *in vitro* studies difficult. For this reason the field of chemical carcinogenesis has been greatly stimulated by work on alkylating agents not requiring metabolic activation. While the chemical reactions are complex, their elucidation was possible, since directly acting agents, such as alkyl sulfates, alkylalkane sulfonates, and certain *N*-nitroso compounds (Fig. 1), could be first studied in the test tube. It was particularly important to mimic physiological conditions, rather than using methods of classical organic chemistry with extremes of pH, temperature and nonaqueous solvents.

The search for alkyl derivatives generally proceeded from nucleoside to homopolynucleotide to single- and double-stranded polynucleotides. We now know that all ring oxygens and nitrogens (except the sugar attachment site), the 2'O of ribose and phosphodiester linkage can all be alkylated to varying extents, depending on the type of alkylating agent (Fig. 1), the alkyl group, and the degree of hydrogen bonding (*1–7*). In addition, crosslinking occurs with bifunctional agents such as mustards (*7*). Simple lactones and epoxides (e.g., ethylene oxide, β-propiolactone) (Fig. 1) are monofunctional alkylating agents, but when carrying an additional functional group they can both crosslink and form cyclic derivatives (*7*). Proteins, lipids, and polysaccharides also contain functional groups reacting with alkylating agents, but little attention to these has been given by biologists.

Chemical Reactions

The chemical structures of nucleoside derivatives found with alkyl sulfates and *N*-nitroso compounds are shown in Figure 2. Of these, all but four have been identified *in vivo* after administration of methyl- or ethylnitrosourea (*1,2,5–7*). It is interesting to note that re-

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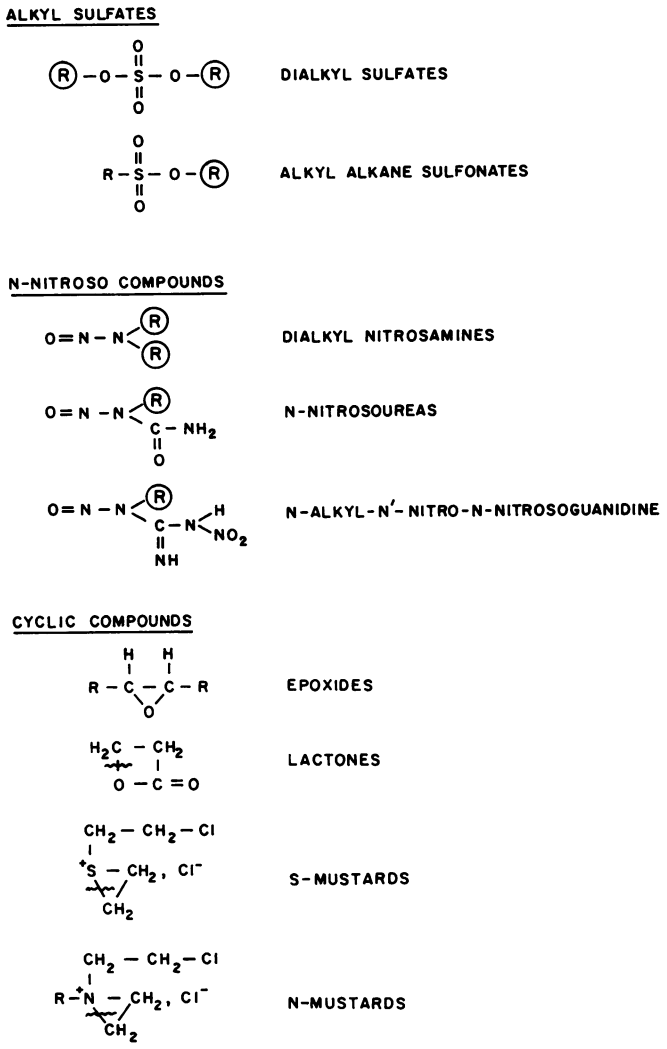

FIGURE 1. Structural formulas of simple alkylating agents. All are direct acting except dialkyl nitrosamines, which must be metabolically activated. The cyclic compounds owe their alkylating ability to reactive, unstable ring structures.

action of the exocyclic amino groups has not been detected in double-stranded nucleic acids *in vivo*, quite in contrast to their ability to react with aromatic compounds (*8*). The other site not found modified, the N-1 of G, is not only hydrogen-bonded but is quite unreactive even in guanosine, except in nonaqueous solution (*9,10*).

The relative mutagenicity and/or carcinogenicity of the various simple alkylating agents appears to be correlated with the ability to react with oxygens. The specificities of eight reagents toward oxygen is ethyl-nitrosourea, ethylnitrosoguanidine > methylnitrosourea, methylnitrosoguanidine > ethyl methanesulfonate > methyl methanesulfonate > diethyl sulfate > dimethyl sulfate (*7*). It can be noted that ethylating agents are "better" carcinogens than the analogous methylating agents; yet, the absolute extent of ethylation is very much less than that of methylation, *in vitro* and *in vivo*.

An important class of chemicals, namely, dialkyl nitrosamines (Fig. 1) and dialkyl hydrazines (R-NH-NH-R), become active alkylating agents, generating an alkyldiazonium ion only as a consequence of metabolic activation. Data for their reactions with nucleic acids *in vivo* are very similar to those obtained using alkyl nitrosoureas (*6*) which directly formed an alkyldiazonium ion ($R\text{-}N_2^+$) by a hydroxyl ion-catalyzed decomposition which occurs rapidly even at neutral pH. All tissues appear to be alkylated by nitrosoureas with virtually the same efficiency, whereas the extent and site of alkylation by alkylnitrosamines or dialkylhydrazines reflects the extent of metabolism in a given cell. Figure 3 presents composite data for the extent of alkylation at each site, *in vivo*, after a single administration of the carcinogen. Here it can be seen that there is a dramatic difference between methylation and ethylation in the proportions of O- and N-alkylation. However, the absolute extent of methylation is 10 to 20-fold that of ethylation *11*).

Having established that at least 11 base derivatives and 16 phosphotriesters can be formed *in vivo* by *N*-nitroso carcinogens, the question becomes that of the biological consequences of each nucleic acid modification. Or, to phrase it another way, how does the cell handle damage?

First, the N-3 and N-7 alkylpurines are depurinated, at differing rates, as a consequence of the instability of their glycosyl bonds, even at pH 7. These rates have been determined *in vitro*, using alkylated DNA, since the stability of the glycosyl bond is greatly increased in the polymer. The half-lives in DNA range from 3 hr for N^7-methyl-deoxyadenosine (7-MedAdo) to 6.5 hr for N^3-methyl-deoxyadenosine (3-MedAdo) to ~155 hr for N^7-methyl-deoxyguanosine (7-MedGuo) (*7*). Thus, without enzymatic action, depurination of these bases occurs at rates 10^6–10^7 greater than unmodified purines. The three most unstable purines (3-alkyl A and G, 7-alkyl A) have been isolated from DNA only as bases. Depyrimidination of the most labile pyrimidine, O^2-alkyl dC, occurs with $t_{1/2}$ estimated to be 35-fold slower than that of 7-alkyl dG (*12*). Nevertheless, since the glycosyl bonds of pyrimidines are two orders of magnitude more stable than those of the purines, the depyrimidination of O^2-alkyl dC can contribute significantly to the formation of apyrimidinic sites. An additional chemical property affecting persistence is -OH-catalyzed imidazole-ring opening of 7-alkyl dG or 7-alkyl dA, measurable at pH 7. This secondarily derived adduct is formed *in vivo* but is not generally quantitated.

Repair Enzymes

Until recently, it was believed that only 3-alkyl A and O^6-alkyl G were enzymatically repaired in mammalian cells. A number of laboratories studying mammalian repair isolated and characterized what is termed 3-methyladenine-DNA glycosylase and O^6-methylguanine-DNA methyltransferase (*6*). In each case, the enzyme is named according to its mode of action and the

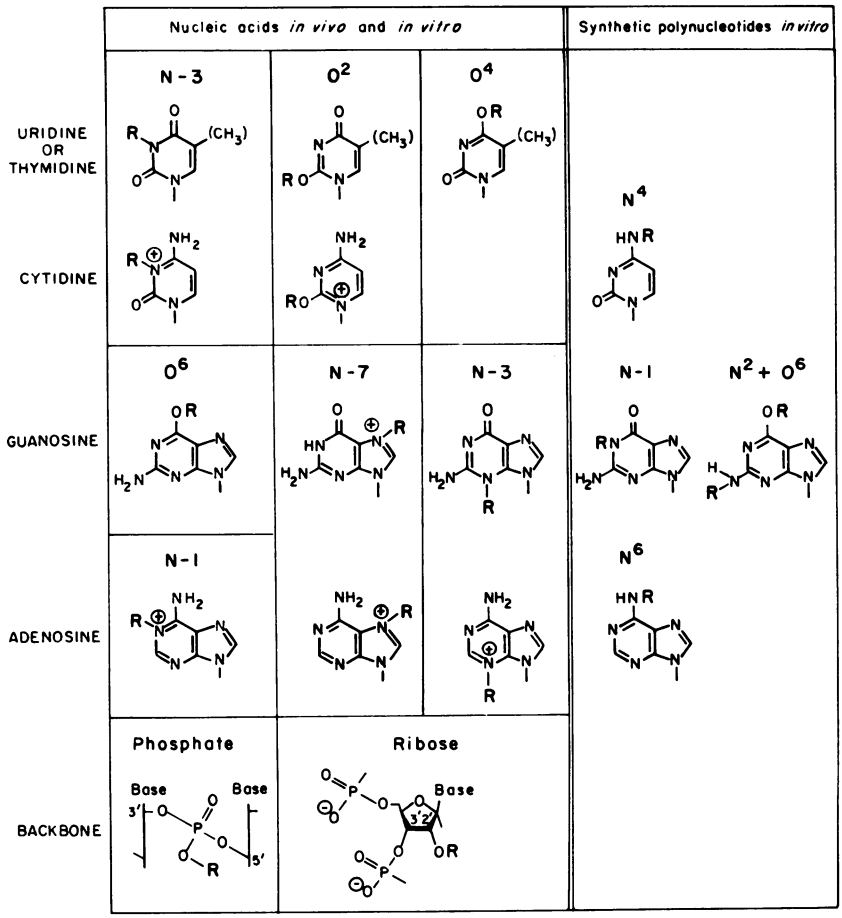

FIGURE 2. (Left) Sites of reaction of simple alkylating agents with nucleic acids. (Right) Reaction at the N^4 of C, N-1 of G, N^2 of G, and N^6 of A has only been demonstrated in polyribonucleotides.

substrate used. However, it was found that *in vivo* many more derivatives were removed/repaired, although for all repair studied the extent was cell or organ specific. Figure 4 illustrates the loss, corrected for cell division, of seven ethyl derivatives from SV-40 transformed human fibroblasts. Over the 75-hr period studied, chemically stable adducts (O-ethyl) had $t_{1/2}$values of 30 to 50 hr, while those with unstable glycosyl bonds (3-EtA, 7-EtG) were removed at a rate considerably greater (3-fold) than their chemical half-life (*13*).

The chemically stable *N*-alkyl derivatives (1-alkyl A, 3-alkyl C, and 3-alkyl T) can form only when these positions are not hydrogen-bonded. Their occurrence in DNA *in vivo* (Fig. 3) either indicates thermal denaturation or that they form in single stranded replicating DNA. Very little is known regarding their repair, but from such limited data, it appears that 1-MeA is persistent(*14*). This adduct, as well as numerous others, can cause errors in replication as will be discussed in a later section.

In Vitro Studies

These findings and others focusing on the alkylpurines first led to a restudy of glycosylase activities toward the other 3- and 7-alkyl purines (*15,16*). Table 1 shows that the enzyme fraction depurinating 3-MeA also acts to a lesser extent on 3-EtA. Similarly, 7-MeG, 3-MeG, and the ethyl analogs are depurinated. There is also evidence that 7-alkyl A is recognized by the glycosylase, but its chemical instability makes this determination less certain. Neither O^6-alkyl G nor 1-alkyl A is a substrate. A calculation of the number of moles of 3-MeA and 7-MeG released by the enzyme from human lymphoblasts, indicates that, after correcting for chemical depurination, 7-MeG is depurinated at half the rate of 3-MeA. This relative affinity for these two alkylpurines is not universal, since the glycosylases from mouse, rat, or gerbil liver appear to have differing affinities toward 7-MeG and 3-MeA (*17*). Whether this indicates that there are two (or more) glycosylase activities, as found in *E. coli*, (*18*) is not established. Gallagher and Brent (*19*) purified a 3-methyladenine-DNA glycosylase from human placenta and report minor activity (~ 8%) toward 7-MeG. Since the affinity for the three substrates measured (3-MeA, 7-MeG, 3-MeG) did not change during purification, they conclude that the three activities are associated with a single glycosylase.

Rodent liver extracts also contain glycosylase activity able to remove ring-opened 7-MeG (rom^{7}G) from DNA (*10*). Once the imidazole ring of 7-alkyl G is open, the

derivative is no longer a quaternary base and the glycosyl bond becomes as stable as that of a normal purine. It is therefore chemically different from the other substrates and it seems likely that repair of rom^{7}G is carried out by a separate enzyme. Such an activity, free of glycosylase activities toward 3-MeA, 7-MeG, or uracil, has been purified from extracts of *E. coli* HB 1100 endo (*20*).

Much has been written on the numerous experiments studying dealkylation by an activity which transfers the alkyl group of O^6-alkyl G to a cysteine of an acceptor protein. The induced bacterial enzyme has been reported to dealkylate O^4-MeT as well (*21–23*). This is not true for the rat liver enzyme (*24*). The experiments on *in vivo* persistence of various alkyl products support the concept that multiple enzymes are required for repair of *O*-alkyl derivatives.

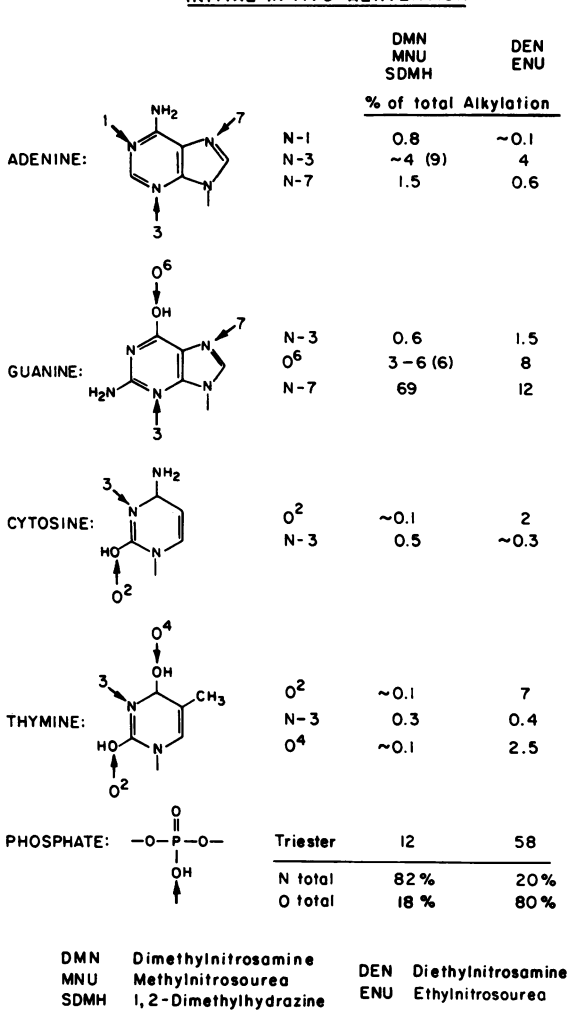

FIGURE 3. The arrows on the structural formulas indicate sites of modification of nucleic acids. The proportion of alkylation at each site is shown for both methylating and ethylating agents. The numbers in parentheses are the expected alkylation, if no repair occurs.

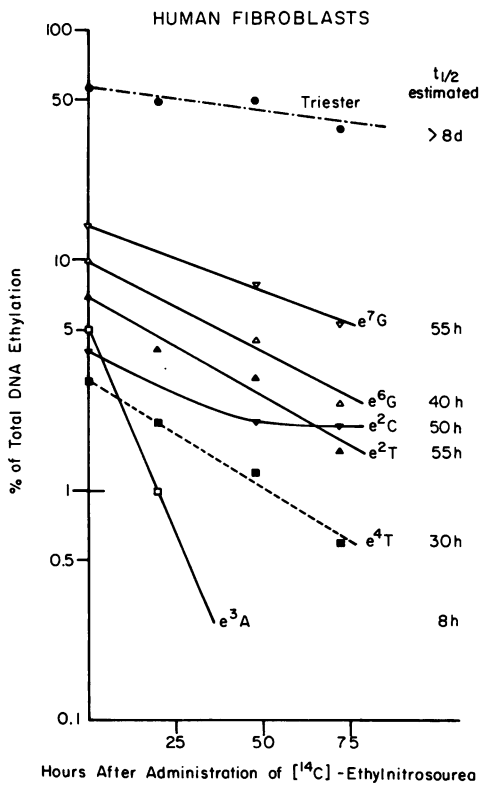

FIGURE 4. Amounts of [^{14}C]ethyl products from ethyl nitrosourea-treated mammalian cells, prelabeled with ^{3}H. The initial proportion of total ethylation is shown on the log scale. All values have been corrected for cell division. The estimated $t_{1/2}$ of derivatives is shown in the figure. Adapted from Bodell et al. (*14*).

In Vivo Studies

A number of laboratories have systematically studied persistence of certain alkyl adducts as a function of dose, size of alkyl group, organ, or cell type. In addition, there are other variables such as age and species which affect the site of tumors. This latter point is illustrated in Table 2.

Human cells in culture also exhibit varied repair rates, e.g., xeroderma pigmentosum (XP) fibroblasts repair O^6-MeG or O^6-EtG less efficiently than normal fibroblasts (*14,15*), while Chinese hamster ovary cells repair neither O^6-alkyl G, but are competent in repair of 3-alkyl A and 7-alkyl G (*26*). Nomenclature has been adopted to identify cell lines which are methyl repair efficient or inefficient (mer$^+$, mex$^+$; mer$^+$, mex$^-$). All these categories are based on repair of 3-MeA, 7-MeG, and O^6-MeG and therefore parallel the corresponding enzymatic activities.

When persistence of a wider range of derivatives is studied, we confirm that human fibroblasts (GM 636) are more efficient in repair of O^6-EtG than are XP fibroblasts (XP-12), but all other ethyl adducts are removed from both cells to approximately the same extent (Table 3). The half-lives in normal cells are all about the same (30-55 hr), except for the very slow loss of triesters

Table 1. Release by human lymphoblasts of methylated or ethylated purines from alkylated deoxypolynucleotides.[a]

Substrate[b]	Depurination, %					
	m^3A	m^7G	m^3G	e^3A	e^7G	e^3G
Me poly(dA-dT)	100					
Me poly(dG-dC)		7	40			
Me DNA	91	6				
Et (poly(dA)•poly-(dT)				26		
Et DNA				39	5	26

[a] Data from Singer and Brent (*15*). Neither 1-alkyladenine nor O^6-alkylguanine was found as a product of glycosylase action.
[b] Me, methylated; Et, ethylated.

Table 2. Effect of single exposure to ethylnitrosoruea.

Animals	Primary site(s) of tumors
Perinatal	
Rat	Brain, central nervous system
Mouse	Lung, liver, kidney
Syrian hamster	Peripheral nervous system
Rabbit	Nervous system, kidney
Gerbil	Melanocytes
Adult	
Rat	Brain, hemopoietic system, kidney
Mouse (M)	Lymph nodes, lung
Mouse (F)	Liver, kidney, lung
Syrian hamster	Forestomach
Rabbit	Kidney
Gerbil	Melanocytes

(Fig. 4). This experiment shows that (1) O-ethyl pyrimidines are repaired and (1) that O^6-EtG repair is independent of repair of other O-ethyl derivatives. A decrease in ethyl triesters, attributed to deethylation, was also reported by Miller et al. (*27*), who incorporated a trinucleotide ethyl phosphotriester in transformed Syrian hamster fibroblasts.

When 10-day-old BD-IX rats are given a single injection of ethylnitrosourea, tumors will eventually develop in the brain (*28*). This organotropy has been attributed to persistence of O^6-EtG. When persistence of seven derivatives is studied in liver, brain, and a pool of other organs, we find that there is evidence that 7-EtG, O^2-EtC, O^4-EtT, O^2-EtT, O^6-EtG and 3-EtA are lost over a 75-hr period with varying half-lives (*30*) (Fig. 5). The brain does not repair any of these derivatives well; particularly, there is no detectable repair of O^4-EtT. Repair in the DNA from "remaining tissue" resembles that of the brain, indicating that in most rat tissues the derivatives which are termed "promutagenic" remain in the DNA for long periods. Only the liver has substantial repair capacity, as also indicated by the relative content/cell of O^6-methylguanine-DNA methyltransferase molecules in rat hepatocytes (60,000), kidney (12,000), and brain (1500) (*31*).

Although not quantitated in a precise manner, Figure 6 illustrates that 7-MeG glycosylase activity (corrected for chemical hydrolysis) is present in brain and liver, but not to any appreciable extent in other tissues, while 3-EtA glycosylase (corrected for chemical hydrolysis) activity appears highest in the brain. These data would also support the concept that there are two glycosylases and their activities do not parallel each other or that of the methyltransferase.

Effect of Size of Alkyl Group

In vitro, repair enzymes are more efficient with methyl than with ethyl substrates (Table 1). Similar conclusions have been reached *in vivo* by Pegg and colleagues studying O^6-alkyl G repair (*32*) and Swenberg and colleagues studying O^4-alkyl T repair (Swenberg et al., personal communication, 1984). Ethyl phosphotriesters are repaired very slowly (Figs. 4 and 5), and a long-term study comparing persistence of methyl and ethyl triesters in mouse liver shows that the ethyl is about seven times more persistent than the methyl (*34*) (Fig. 7). Extrapolation is usually dangerous. Nevertheless, as more data are obtained it is likely that the rate of repair will be found to diminish with the size of the substituent, until the substituent is no longer recognized by the same enzyme but possibly by one repairing bulky adducts.

The net effect is that, while ethylation per carcinogenic dose produces fewer of each derivative than does methylation (*31*), the decreased repair of ethyl adducts (or higher homologs) can compensate for this. Over a period of time, ethyl adducts can accumulate or persist during replication.

Effect of Alkyl Derivatives on Transcription and Replication

It is a widely held hypothesis that initiation of carcinogenesis is a consequence of a base change in progeny nucleic acids. One likely mechanism is through point

Table 3. Persistence of ethyl derivatives.[a]

Fibroblast	Loss after 48 hr cell culture, %						
	O^6-EtG	O^2-EtC	O^2-EtT	O^4-EtT	7-EtG	3-EtA	Triester
GM 637	55	48	40	55	43	100[b]	13
XP-12	14	68	42	60	46	100[b]	7
In vitro	0	0	0	0	19	65	0

[a] Data from Bodell et al. (*14*). Cells were prelabeled with [^{3}H]thymidine 24 hr, then treated with [^{14}C]ethylnitrosourea for 1 hr. The total extent of ethylation at the end of 1 hr was 1–4 ethyls/10^5 DNA-P.
[b] 80% loss in 20 hr cell culture.

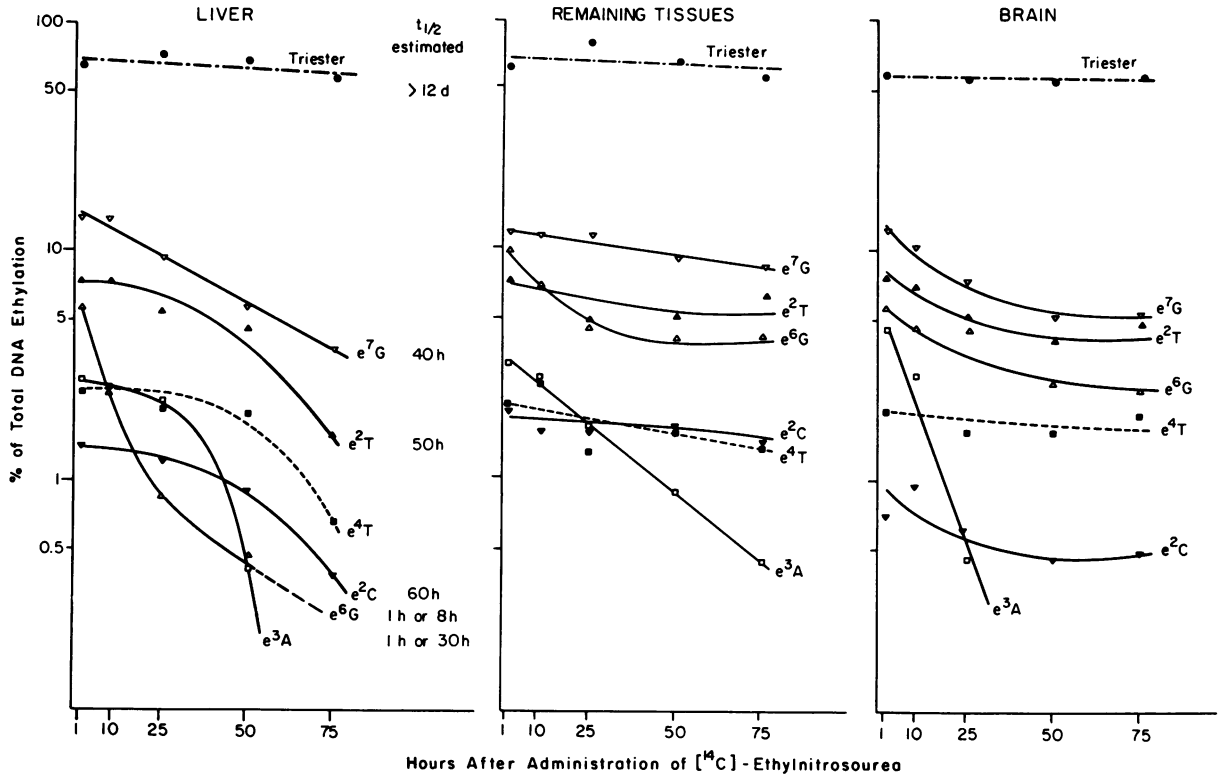

FIGURE 5. Amounts of ethyl derivatives at 1, 10, 25, 50, and 75 hr after administration of [^{14}C]ethylnitrosourea to 10-day-old BD IX rats. The number of ethyl groups bound in various organs was 1.3–3 per 10^5 DNA-P (*30*): (left) liver DNA; estimated half-lives of each derivative are shown in the figure; (middle) pooled DNA from spleen, lung, thymus, muscle, and intestine; (right) brain DNA.

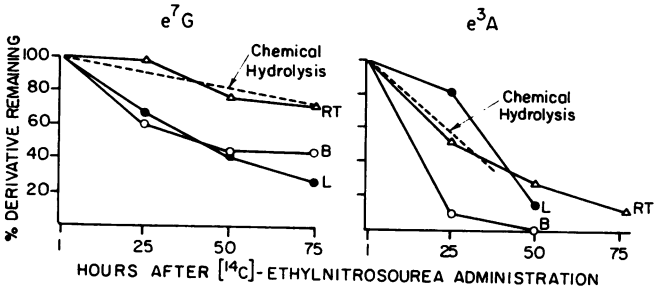

FIGURE 6. Stability of e^7G (7-ethyl G) and e^3A (3-ethyl A) in DNAs in ethylnitrosourea-treated 10-day-old BD IX rats (*30*), as compared to the chemical stability *in vitro* (*7*): (--) rate of chemical hydrolysis at pH 7, 37°C; (○) brain; (●) liver; (Δ) other tissues, pooled.

mutation. The list of reactions capable of causing such change is long, including cleavage of the glycosyl bond leading to apurinic and apyrimidinic sites (AP sites) (*35*), changes in tautomeric equilibrium (*36,37*) and ionization, and addition of alkyl groups to base nitrogens and oxygens (*7*). Both replicating and transcribing enzymes have been used *in vitro* to ascertain such changes.

Table 4 shows representative data for the efficiency and type of bases incorporated in transcription of homopolynucleotides containing a single type of modified base. Two of these bases, N^4-methoxy C and N^6-methoxy A, are products of methoxyamine reaction (*38,39*), not alkylation products, but illustrate the specificity of tran-

Table 4. Fidelity in transcription of polynucleotides containing promutagenic bases.[a]

Modified base in ribopolymer	Frequency of incorporation, NTP/modified base			
	C	A	U	G
N^4-Methoxy C	ND	1/1.1	ND	ND
O^4-Methyl U		1/3	ND	1/3
N^6-Methoxy A	1/30	ND	1/3	—
O^6-Methyl G	1/14	1/9	1/4	—

[a] Data are from nearest-neighbor analyses after transcription using *E. coli* DNA-dependent RNA polymerase in the presence of 10 mM $MgCl_2$ and equimolar amount of all four rNTPs with GTP [α^{32}P] labeled. The modified bases represented 2–17% in copolymers with C, A, or U.

Table 5. Fidelity in replication with different polymerases.[a]

Polymer	Frequency of misincorporation, dGTP/modified base		
	Pol I	Klenow fragment	Reverse transcriptase
Poly d(A, ϵA)[b]	1/433	1/550	1/25
Poly d(A, O^4-MeT)	1/12		1/3
Poly d(A, C)	1/1.6		1/1

[a] Data from Singer et al (*48*). O^4-MedTTP substituted for dTTP in Pol I catalyzed synthesis using a poly d[A-T] primer/template. The resulting polymer, poly d[A-T, O^4MeT], when replicated, increased the dGTP misincorporation 3-fold (*43*).

[b] ϵA is 1,N^6-ethenodeoxyadenosine.

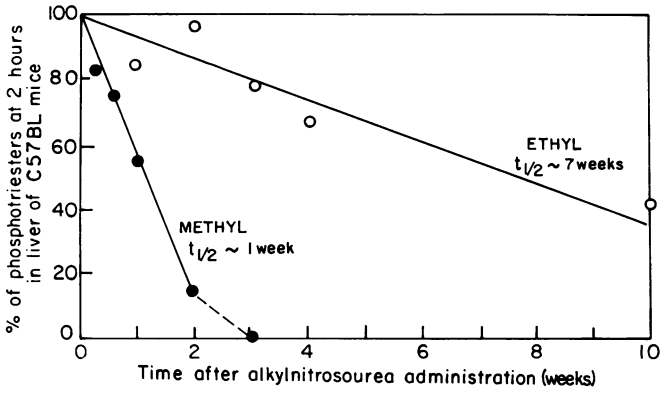

FIGURE 7. Loss of methyl and ethyl phosphotriesters from liver DNA of C57BL mice given a single injection of methylnitrosourea or ethylnitrosourea. Phosphotriesters were determined from the extent of degradation induced in isolated DNA by alkali. Data from Shooter and Slade (*34*).

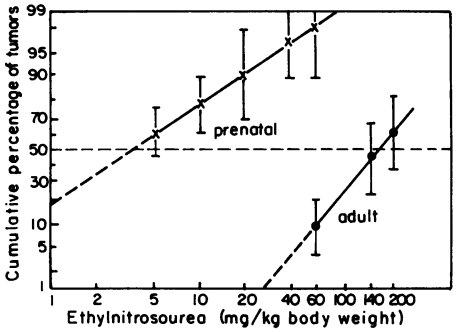

FIGURE 8. Dose-response relationships for the prenatal induction of malignant tumors of the nervous system compared with that in adult rats. Ethylnitrosourea was administered as a single dose. Adapted from Druckrey et al. (*28*).

scription, since both have specifically changed tautomeric equilibria, reflected in the transcript. The other two bases, O^6-alkyl G and O^4-alkyl T, found *in vivo* after alkylation and persistent in certain organs and cells (*13,30,33,40*), also direct substantial misincorporation. Similar data have been found for O^2-alkyl T and O^2-alkyl C in various systems testing mutation (*41–43*). The *N*-alkyl derivatives modified on the N-1 of A and N-3 of C and U/T are similarly mutagenic *44,45*), but since the Watson-Crick hydrogen-bonding sites are unavailable and no base pair can be formed, they resemble AP sites in a somewhat random misincorporation of any base, although AP sites are often bypassed or stop replication (*46,47*). It is likely that the polymerizing enzymes play a role in some discrimination of misincorporation.

Replication with a polymerase capable of correcting errors, DNA polymerase I (Pol I), reduces the number of misincorporated bases. Nevertheless, O^4-MeT directs a considerable amount of dGTP (Table 5). When any possibility of hydrogen bonding is lost, e.g., 1, N^6-etheno A, Pol I does not significantly cause misincorporation; but since replication is not appreciably hindered, it is likely that the bulky derivative is bypassed (*48*). A less discriminating enzyme, reverse transcriptase from avian myeloblastosis virus, markedly increases misincorporation, resembling transcription which also is unable to correct insertion of a nonpairing base.

These *in vitro* experiments lead us to hypothesize that errors, or point mutation, can result from the presence of almost all alkyl derivatives.

Assessment of Exposure to Alkylating Agents

In previous sections of this paper, it was noted that the extent and proportion of derivatives resulting from alkylation of DNA is dependent on the agent, organ, and species. Persistence is a function of both chemical instability and repair enzymes; the latter varying again with organ and species. To date, it has not been possible unequivocably to equate the presence or persistence of a single derivative with the site of ultimate tumors (*31*), in spite of the fact that most derivatives have the potential to cause mutation. In addition, the age of an animal is a major variable (Figure 8) and is likely to be so with humans. It is therefore difficult to envision how quantitation of even a majority of alkyl products can be used to predict exposure leading to malignancy.

Given the limited data on exposure versus cancer incidence in man, it becomes clear that additional insights into parameters correlating with carcinogenesis are needed, as well as the determinants of transformation.

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